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ARCHITECTURE OF HAPTORAL MUSCULATURE IN THREE SPECIES OF *LIGOPHORUS* (MONOGENEA, ANCYROCEPHALIDAE)

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ABSTRACT

The posterior attachment organs of monogeneans known as haptors show exceptional morphological plasticity and play a major role in monogenean taxonomy. Our knowledge of haptoral musculature, however, is still very limited and further study of this musculature in different groups of monogeneans may be instrumental in improving their taxonomic system and furthering our knowledge of their modes of attachment. This study used phalloidin staining in conjunction with confocal microscopy to examine haptoral musculature in three species of *Ligophorus* (*L. llewelyni* Dmitrieva et al., 2007*, L. pilengas* Sarabeev et Balbuena, 2004 and *L. kaohsianghsieni* (Gusev, 1962) Gusev, 1985), a member of the monopisthocotylean family Ancyrocephalidae *sensu* Bychovsky et Nagibina, 1978. Sclerotised structures (anchors, connecting bars and marginal hooks) were visualised simultaneously with muscles by capturing laser light reflected off the surfaces of these sclerites. The haptoral musculature was shown to be identical in *L. pilengas* and *L. llewelyni* and essentially similar between these two species and *L. kaohsianghsieni,* but the latter species had also some notable differences. Common to all three species were thick extrinsic muscles arising from the inner roots of the anchors, a muscle bundle connecting the inner roots of the opposite ventral anchors, muscles running from the outer roots of the anchors to the body wall, muscles originating from the dorsal bar and inserting on the anchoral openings and muscles connecting the bars with anchoral roots. *Ligophorus llewelyni* and *L. pilengas* have a more elaborate haptoral musculature than *L. kaohsianghsieni* with some additional muscles, tendon-like muscle extensions and a strong bracket-shaped muscle presumably functioning as a catch-muscle for the dorsal anchors. Functional roles of individual haptoral muscles in attachment are discussed and the architecture of haptoral musculature in *Ligophorus* is compared with that of other ancyrocephalids.

Key words: confocal microscopy, haptor, *Ligophorus,* Monogenea, musculature, phalloidin staining

ОРГАНИЗАЦИЯ МУСКУЛАТУРЫ ПРИКРЕПИТЕЛЬНОГО ДИСКА У ТРЕХ ВИДОВ ИЗ РОДА *LIGOPHORUS* (MONOGENEA, ANCYROCEPHALIDAE)

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РЕЗЮМЕ

Хотя прикрепительные диски моногеней отличаются исключительным морфологическим разнообразием и играют значительную роль в систематике моногеней, организация их мускулатуры все еще остается недостаточно изученной. Дальнейшее исследование этой мускулатуры в разных группах моногеней позволит уточ-

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нить их таксономическую систему и улучшит понимание способов их прикрепления к хозяевам. В статье описаны результаты исследования мускулатуры прикрепительного диска с использованием окраски фаллоидином и конфокальной микроскопии у трех представителей рода *Ligophorus* (*L. llewelyni* Dmitrieva et al., 2007, *L. pilengas* Sarabeev et Balbuena, 2004 и *L. kaohsianghsieni* (Gusev, 1962) Gusev, 1985), относящегося к семейству Ancyrocephalidae *sensu* Bychovsky et Nagibina, 1978 (Monopisthocotylea). Склериты (срединные крючья, соединительные пластинки и краевые крючья) были исследованы одновременно с мышцами в отраженном свете лазера. Исследование показало, что мускулатура прикрепительных дисков идентична у *L. pilengas* и *L. llewelyni* и в целом сходна у этих двух видов и *L. kaohsianghsieni,* но для последнего вида также характерен ряд отличий. Общими для всех трех видов являются мощные внешние мышцы, отходящие от внутренних отростков срединных крючьев, мышечный пучок, соединяющий внутренние отростки противоположных вентральных срединных крючьев, мышцы, идущие от наружных отростков к стенке тела, мышцы, отходящие от дорсальной пластинки к отверстиям срединных крючьев, и мышцы, соединяющие отростки срединных крючьев и пластинки. Мышечная система прикрепительного диска *L. llewelyni* и *L. pilengas* устроена сложнее, чем у *L. kaohsianghsieni,* и отличается рядом дополнительных мышц, сухожилиеподобными участками на концах мышц и мощной V-образной мышцей, предположительно участвующей в фиксации дорсальных крючьев. В статье обсуждается функциональная роль отдельных мышц прикрепительного диска, и проводится сравнение прикрепительной мускулатурой у *Ligophorus* и у других представителей Ancyrocephalidae.

Ключевые слова: конфокальная микроскопия, прикрепительный диск, *Ligophorus,* Monogenea, мускулатура, окраска фаллоидином

INTRODUCTION

The survival of monogeneans, like that of other ectoparasites, depends on efficiency of their attachment to the host, which is effected primarily by their posterior attachment organ (haptor). Given their adaptive importance, it is not surprising that haptors exhibit exceptional morphological diversity and feature prominently in monogenean taxonomy. The classification of monogeneans, especially monopisthocotyleans, has traditionally relied heavily on general morphology of the haptor and characters of haptoral sclerites, while other aspects of the attachment apparatus, such as the architecture of haptoral musculature, have generally received much less attention. Although in some instances, the musculature of haptors was shown to be sufficiently variable to be used as a source of taxonomically informative characters (see, for instance, Llewellyn 1960 for Amphibdellidae and Gerasev 1977, 1981, 1989 for *Dactylogyrus*), in most cases its morphology is too poorly known to be of much value for phylogenetic analysis. One of the main reasons is a remarkable complexity of this musculature, which makes it challenging to study using conventional light microscopy.

The use of fluorescently tagged phalloidin and other phallotoxins as actin-specific probes in conjunction with confocal microscopy allows for an accurate three-dimensional visualisation of myoanatomy, which helps overcome some of the limitations of conventional methods. Phallotoxin staining can be especially useful in studying haptoral musculature in monopisthocotyleans that have an undivided haptor typically with relatively few associated muscles. There have been a number of confocal microscopy studies of monogenean musculature (Halton et al. 1998; Zurawski et al. 2001, 2003; El-Naggar et al. 2004, 2007; Halton and Maule 2004; Arafa et al. 2007; Valigurová et al. 2011), but among more than 20 families of the Monopisthocotylea the haptoral muscles have been studied by phalloidin fluorescence only in a few species of Gyrodactylidae (El-Naggar et al. 2004, 2007; Arafa et al. 2007). It is clear that further confocal studies of haptoral musculature on a wider range of monopisthocotyleans may provide abundant information for phylogenetic analysis and may improve our understanding of the functional role of individual muscles in attachment.

Members of the family Ancyrocephalidae *sensu* Bychovsky et Nagibina, 1978 (or subfamily Ancyrocephalinae *sensu* Kritsky et Boeger, 1989) deserve a particular interest in this respect as the molecular phylogenetic analysis raises the possibility that these monopisthocotyleans might be polyphyletic with two or three separate clades (Simková et al. 2003, 2006; Plaisance et al. 2005). The purpose of this study was to examine the arrangement of haptoral muscles in *Ligophorus,* an ancyrocephalid living on the gills of

several species of mullets (fam. Mugilidae). The musculature of three species of *Ligophorus* was studied using phalloidin staining and confocal microscopy and an attempt was made to use confocal microscopy for the simultaneous visualisation of muscles and sclerotised haptoral structures (anchors, connecting bars and marginal hooks).

Accurate identification of muscle attachment sites on sclerites is crucial for correct reconstruction of haptoral musculature. Recently, chromotrope 2R was successfully used in combination with phalloidin to double-stain sclerites and muscles for confocal microscopy (Garcia-Vasquez et al. 2012). As a prolonged staining with chromotrope 2R (usually, for a period of 1–2 days) may compromise the quality of phalloidin preparations, the present study employed an alternative approach: images of haptoral armament were acquired by capturing laser light reflected off the surfaces of sclerites. The anchors and marginal hooks proved to be more reflective than the connecting bars, but the bars were more homogeneously opaque and more easily visualized by their appearance as shadows in the confocal images. Since the sclerites of *Ligophorus* are well characterised in the literature, we provide in this paper only their brief description focusing on the characteristics of particular relevance to muscle architecture.

MATERIAL AND METHODS

Ligophorus species were collected from three specimens of the redlip mullet (*Liza haematocheilus* Temminck et Schlegel, 1845), 29–38 cm in total length, caught in the Strait of Kerch off the coast of the Crimean Peninsula (Black Sea: 45°07´N 36°25´E). Three species were identified from the 27 individuals collected: *L. llewelyni* Dmitrieva et al., 2007, *L. pilengas* Sarabeev et Balbuena, 2004 and *L. kaohsianghsieni* (Gusev, 1962) Gusev, 1985.

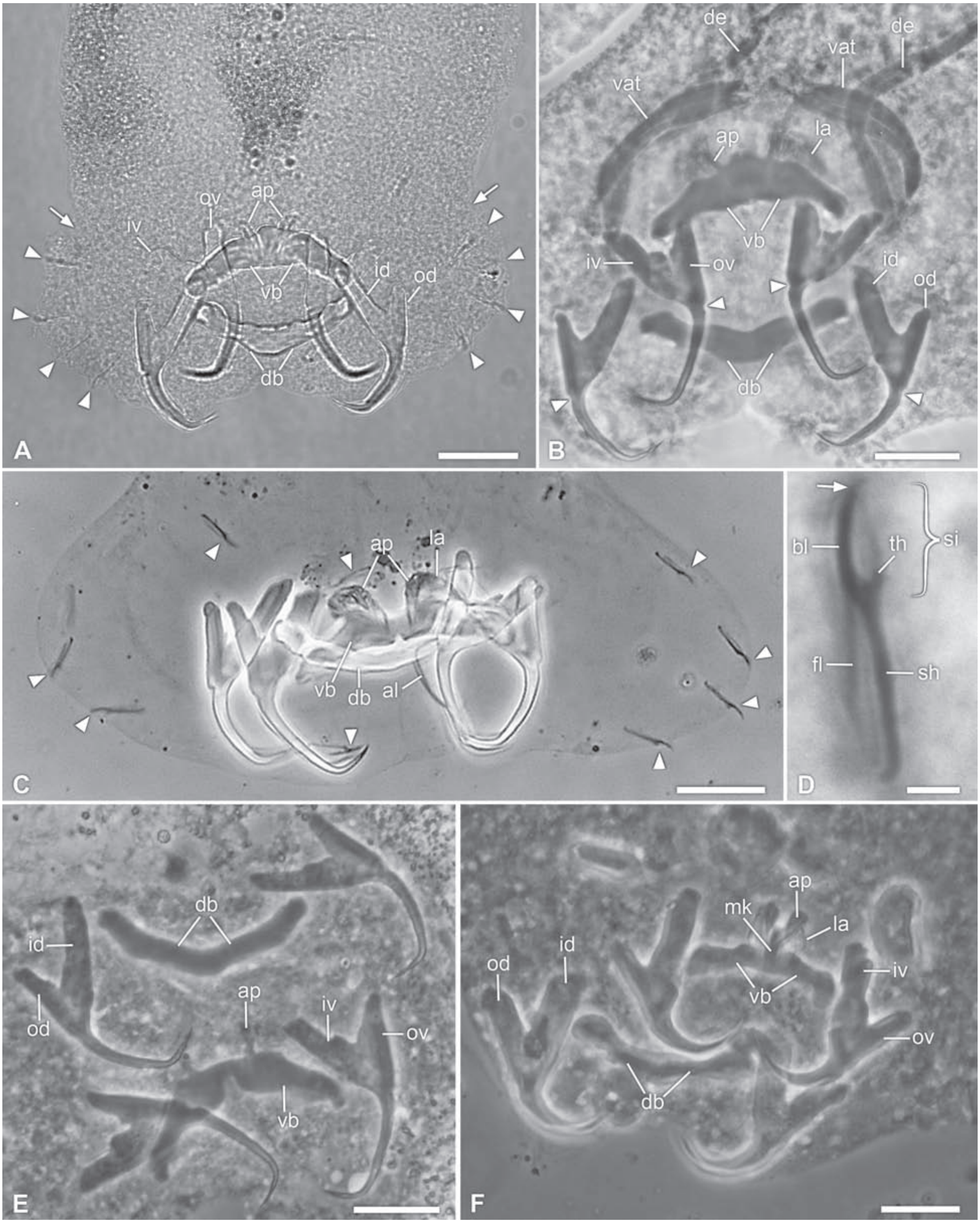
The specimens of *Ligophorus* were flat-fixed under a coverslip at ambient temperature for 8 h with 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS) and were kept at 4 °C in 0.01M PBS containing 0.1% sodium azide. All subsequent steps were performed at room temperature. The worms were permeabilized for 2 h with 0.5% Triton X-100 in PBS, stained 2–3 h with tetramethylrhodamine B isothiocyanate (TRITC) conjugated phalloidin (Sigma-Aldrich, P1951), rinsed in PBS and then mounted on slides with Vectashield (Vector Laboratories Inc.). Confocal images were collected on a Leica TCS SP5 confocal laser scanning microscope. Sclerites were visualized in a reflection mode: the animals were irradiated with a He-Ne (633 nm) laser and the images were acquired at a detection range of 630–645 nm. All confocal images of phalloidin-stained whole mounts were presented as maximum intensity projections of the Z-stacks.

RESULTS

Like most ancyrocephalids, *Ligophorus* has a haptor with a dorsal and ventral pairs of anchors, two connecting bars associated with each pair of the anchors, and 7 pairs of marginal hooks (Fig. 1). In *L. llewelyni* and *L. pilengas,* the haptor has about the same width as the posterior trunk (Fig. 1A). In *L. kaohsianghsieni,* the body narrows down posteriorly toward the haptor and the diameter of the haptor is smaller than that of the posterior trunk (Fig. 2D). The haptors of all three species have wing-shaped lateral flaps and haptor's transition to the trunk is often marked by a noticeable constriction (Fig. 1A, *arrows*). The worms of all three species attach to the host wedging their haptors between secondary gill lamellae and driving ventral and dorsal anchors into the tissue of two adjacent lamellae. The anchors have a fish-hook shape typical for ancyrocephalids with two proximal root processes and a curved tapering

Fig. 1. General morphology of haptors and haptoral sclerites in squeeze preparations. Bright field (A) and phase contrast (B–F). A. Posterior trunk and haptor of *L. llewelyni* showing marginal hooks (arrowheads) and a constriction between the trunk and the haptor. B. Haptoral sclerites and muscles of *L. pilengas*. Arrowheads indicate notches on the outer sides of the anchors. C. Haptor in *L. pilengas*. Arrowheads point to marginal hooks. D. Marginal hook in *L. pilengas*. Arrow indicates the position of the hook's opening. E. Sclerites in *L. llewelyni*. F. Sclerites in *L. kaohsianghsieni*. In A–C, E, and F, anterior is towards the top. Scale bars: 20 μm (A–C, E, F), 2 μm (D).

Abbreviations: al – anchoral ligament; ap – anterior process; bl – blade of marginal hook; db – dorsal bar; de – dorsal extrinsic muscles; fl – filament loop; id – inner root of dorsal anchor; iv – inner root of ventral anchor; la – lamina of anterior process; mk – median knoll; od – outer root of dorsal anchor; ov – outer root of ventral anchor; si – sickle of marginal hook; sh – shaft of marginal hook; th – thumb of sickle; vat – transverse muscle of ventral anchors; vb – ventral bar.


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point (Fig. 1A–C,E,F, 2A–B). The tips of the root processes are shaped as triangular knobs (especially noticeable in *L. kaohsianghsieni*) that serve as attachment sites for anchoral musculature (Fig. 2E, *kiv*). The surfaces of these knobs stain heavily with phalloidin and are readily identifiable in confocal images as bright cup-shaped structures (Fig. 3A,E, *arrowheads*). Each anchor has a notch on its outer side at the transition between the blade and the outer root process (Fig. 1B, *arrowheads*); two thread-like ligaments (Fig. 1C, *al*) extend from this notch to the opening through which the anchor is projected from the haptor (anchoral opening). In attached worms, the points of the ventral anchors are oriented ventrolaterally facing diagonally away from each other. This orientation allows them to gaff the host tissue like a spreader distributing the force over a larger area. The dorsal anchors, in contrast, are angled dorsomedially toward one another and the tissue is clasped between their tips in a pincer-like fashion.

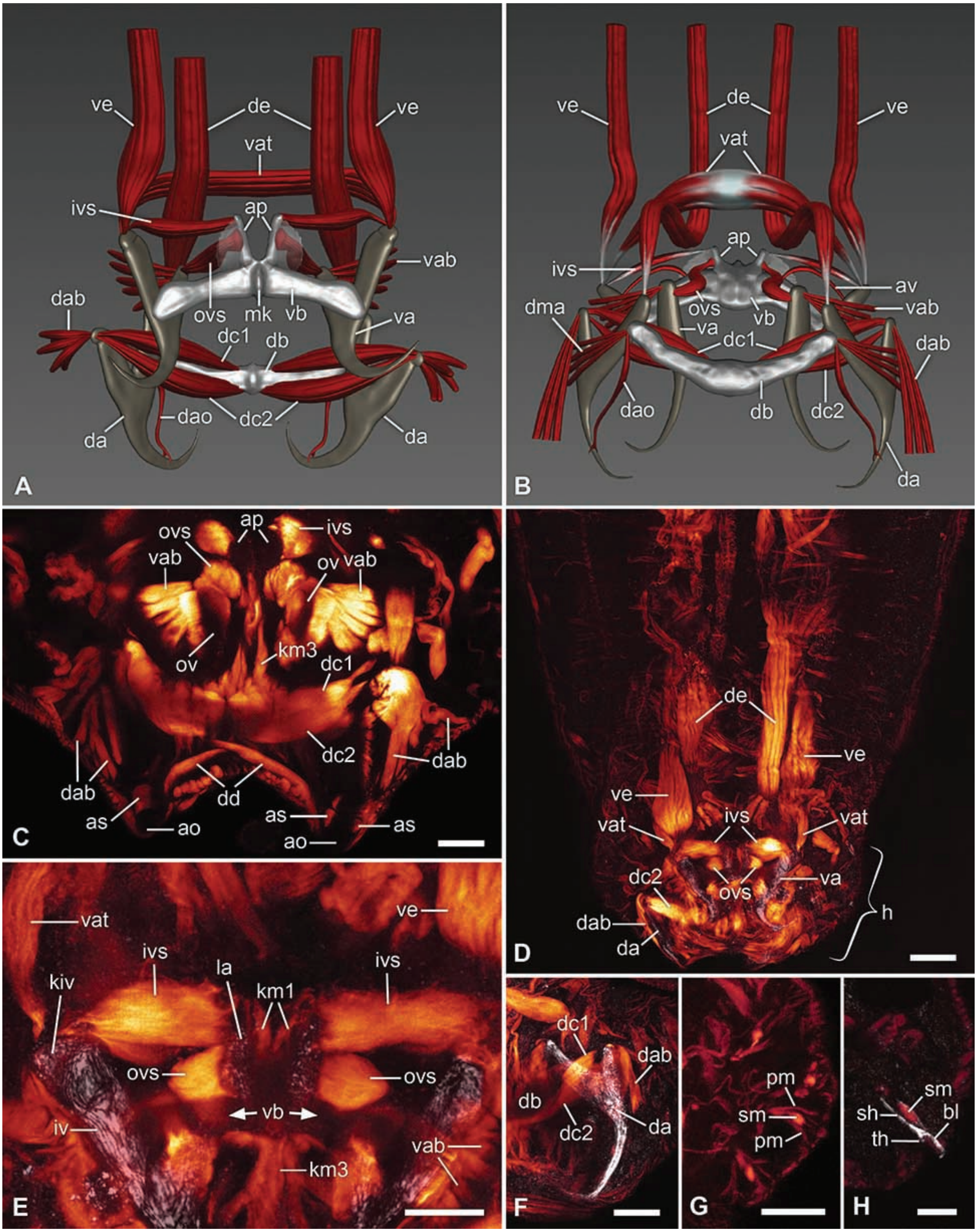
The ventral bar (Fig. 1A–C,E,F, *vb*) is straight or slightly U-shaped and bears a median knoll (Figs. 1F, 2A, 3F, *mk*) on its ventral side and a pair of rod-like submedian processes projecting anteriorly on each side of the median knoll (Figs. 1A–C,E,F, 2A–C, 3C, *ap*). Each anterior process is provided with a wing-shaped lamina that extends from it in a ventrolateral direction (Figs. 1B,C,F, 2E, *la*). The shapes and sizes of anterior processes, laminae and the median knoll are quite variable within and across species, but anterior processes are closer to each other and generally more pronounced in *L. kaohsianghsieni* than in the other two species. The dorsal bar has the shape of a widely spread V in *L. llewelyni* (Fig. 1A,E, *db*) and *L. pilengas* (Fig. 1B,C, *db*) and is almost straight in *L. kaohsianghsieni* (Fig. 1F, *db*). In all three species, the lateral ends of both bars are closely associated with the body wall or embedded into it (Fig. 3B, *arrows*) and are likely to be immobile relative to the body surface. In *L. llewelyni* and *L. pilengas,* the body-wall musculature in front of the dorsal bar forms a grid-like arrangement of thin longitudinal and wide transverse fibres (Fig. 3B, *gb*) and the body wall directly above the bar lacks musculature entirely (Fig. 3B).

The haptoral musculature is identical in *L. llewelyni* and *L. pilengas* and essentially similar between these two species and *L. kaohsianghsieni,* but the latter species has also some notable differences. The following description of haptoral musculature applies to all three species, except where differences between muscles of *L. kaohsianghsieni* and those of the other two species are explicitly stated.

In general, the haptoral muscles in *L. kaohsianghsieni* are thicker than in *L. llewelyni* and *L. pilengas* and the musculature fills more space in the interior of the haptor (compare Figs. 2D and 3A). The musculature of the dorsal anchors (Fig. 4C) has five muscle bundles common to all three species. A wide bundle, the dorsal extrinsic muscle, arises from the tip of the inner root of each anchor (Figs. 1B, 2A,B,D, 3A,C,F, 4C, *de*) and extends anteriorly into the trunk to attach to the dorsal body wall at the posterior margin of the ovary. Three of the bundles originate from the proximal end of the outer root. Two of these are thick bundles (*dc*-muscles) that run medially toward the dorsal bar, one inserting on the anterior side of the mid-portion of the bar (Figs. 2A–C,F, *dc1*) and the other on its posterior side (Figs. 2A–D,F, 3A,E, *dc2*). The fibres of the third bundle (*dab*-bundle) in *L. kaohsianghsieni* diverge in a fan-like manner from a single point on the anchor to insert widely on the dorsal and posterior body wall of the haptor (Fig. 2A,C,D, *dab*). In *L. llewelyni* and *L.*

Fig. 2. Haptoral musculature and sclerites as three-dimensional representations (A, B) and on phalloidin-stained whole mounts (C–H) with sclerites visualised in a reflection mode. A. Haptoral sclerites and musculature of *L. kaohsianghsieni*, ventral view. B. Haptoral sclerites and musculature of *L. llewelyni* and *L. pilengas*, dorsal view. Contractile portions of muscle bundles are shown in red, tendinous sections in white. C. Posterior haptor in *L. kaohsianghsieni*. D. Posterior trunk and haptor in *L. kaohsianghsieni*. E. Mid-portion of the haptor in *L. kaohsianghsieni* showing musculature connected to the ventral bar and anchors. F. Musculature of the dorsal anchor in *L. pilengas*. G. Musculature of marginal hooks in *L. llewelyni*. H. Marginal hook in *L. kaohsianghsieni*. Color-codes in C–H: F-actin (orange), sclerites (white). Anterior is towards the top. Scale bars: 10 µm (C, E, F, G), 20 µm (D), 5 µm (H).

Abbreviations: ao – anchoral opening; ap – anterior process; as – anchoral sphincter; av – arch-shaped muscle; bl – blade of marginal hook; da – dorsal anchor; dab – muscles coming from dorsal anchor to body wall; dao – muscle running from dorsal bar to anchoral opening; db – dorsal bar; dc1–2 – muscles connecting dorsal bar and anchors; dd – diagonal muscles of dorsal bar; de – dorsal extrinsic muscles; dma – muscles connecting dorsal bar to mid-portion of dorsal anchor; h – haptor; ivs – muscle attached to anterior process; kiv – knob on inner root of ventral anchor; km1–3 – muscle fibres attached to median knob of ventral bar; la – lamina of anterior process; mk – median knoll; ov – outer root of ventral anchor; ovs – muscle attached to wing-shaped lamina; pm – protractor muscle of marginal hook; sh – shaft of marginal hook; sm – spindle-shaped muscle of marginal hook; th – thumb of sickle; va – ventral anchor; vab – muscles coming from ventral anchor to body wall; vat – transverse muscle of ventral anchors; vb – ventral bar; ve – ventral extrinsic muscles.


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pilengas, dab-bundles are more compact (Figs. 2B,F, 3A, *dab*) and run posteriorly almost parallel to the outer root of the anchor attaching to the body wall laterally to the anchoral opening. The fifth bundle (*dao*-muscle) associated with the dorsal anchors is very thin and probably consists of one or two myofibres (Figs. 2A,B, 3E, *dao*). This bundle arises from the end of the dorsal bar and courses posteriorly to terminate with two small finger-like branches around the anchoral opening.

Ligophorus llewelyni and *L. pilengas* have a bilateral pair of additional muscle bundles (*dma*-bundles) that appear to be associated with the dorsal bar and each of the dorsal anchors. Each bundle is attached to the lateral end of the bar or possibly to the body wall in the immediate vicinity of the bar (Figs. 2B, 3E, *dma*). From here it fans out laterally and ends with a wide array of fibres between the *dab*-muscles and the outer root of the dorsal anchor. The exact points of attachment of the lateral ends of these bundles cannot be clearly identified.

The musculature of the ventral anchors (Fig. 4A) has five muscle bundles common to all three species. Three of these are attached to the inner roots of each anchor originating from a triangular knob at the distal tip of the root. In *L. llewelyni* and *L. pilengas* these bundles are attached to the knob through relatively long actin-poor tendons (Fig. 2B), but in *L. kaohsianghsieni* the contractile portions of the muscle fibres are in direct contact with the knob (Fig. 2A,E). One of these bundles is a thick extrinsic muscle (Figs. 2A,B,D,E, 3A,F, *ve*) that continues anteriorly through much of the trunk and ends on the ventral body wall immediately posterior to the male copulatory organ. The second bundle (*vat*-muscles, Figs. 1B, 2A,B,D,E, 3A,C,E, *vat*) proceeds from its insertion on the knob in an antero-dorsal direction but then turns and courses transversely in the medial direction to join at the body midline with the same muscle coming from the opposite anchor. In *L. kaohsianghsieni*, *vat*-muscles appear as a single bundle spanning continuously between the opposite ventral anchors (Fig. 2A), but in the other two species they are joined together with actin-poor tendon-like extensions (Fig. 2B) and in phalloidin-stained preparations and under phase contrast they usually appear widely separated from one another (Figs. 1B, 3A, *vat*). These paired muscles in some specimens of *L. llewelyni* and *L. pilengas* (probably where they are in a more contracted state) clamp the dorsal extrinsic muscles folding their posterior portions into a loop (Fig. 1B; also shown graphically in Fig. 2B). The third muscle bundle of the inner root (*ivs*-muscle) extends from the knob in the medial direction and attaches to the tip of the anterior process on the ventral bar (Figs. 2A,B,C–E, 3C,F, *ivs*). In *L. llewelyni* and *L. pilengas,* this bundle is very thin and, because it has long actin-poor tendons at both ends, the precise location of its attachment points on the knob and anterior process cannot be clearly seen.

The remaining two bundles of the ventral anchors common to all three species are attached to their outer roots. The first bundle (*ovs*-muscle) extends from the tip of the root towards the ventral bar and attaches to the base of the wing-shaped lamina close to its connection with the anterior process (Figs. 2A–C,E, 3A,C,F, *ovs*). In all the squeeze preparations studied, this bundle is considerably longer than the distance between the anterior process and the outer root of the ventral anchor and, as the processes are located ventrally to the root, the muscle bundle runs more or less dorsoventrally and is folded in zigzag fashion. The second muscle bundle (*vab*-bundle, Fig. 2A–C,E, *vab*) extends from the outer side of the root somewhat more distally than the first and fans out towards the ventro-lateral wall of the haptor.

In addition to the muscles coming from the anchors, dorsal and ventral bars are connected each to a bilaterally symmetrical pair of muscle bundles extending to the body wall (*dd*- and *dv*-muscles, Figs. 2C, 3E, *dd*, Fig. 3C,F, *dv*). Each of these muscles originates on the posterior side of the bar about halfway between the midpoint and the lateral ends of the bar and courses diagonally in a medio-posterior direction crossing the opposite muscle at the body midline. The posterior ends of the dorsal pair of these muscles insert on the body wall medially to the openings of the dorsal anchors and those of the ventral pair laterally to the ventral anchors close to their mid-portion. The openings of the dorsal pairs of anchors are encircled by wide circular muscle bands (Fig. 2C, *as*).

Another set of muscle bundles is associated with the mid-portion of the ventral bar (*km*-bundles). These bundles consist of a loose assemblage of myofibres arising from or near the median knoll of the bar and running in a trough formed by wing-shaped laminae of anterior processes. These fibres extend forward on either side of the median knoll toward the anterior margin of the bar (Figs. 2E, 3F, *km1*) where they turn around the bar to its dorsal side and then

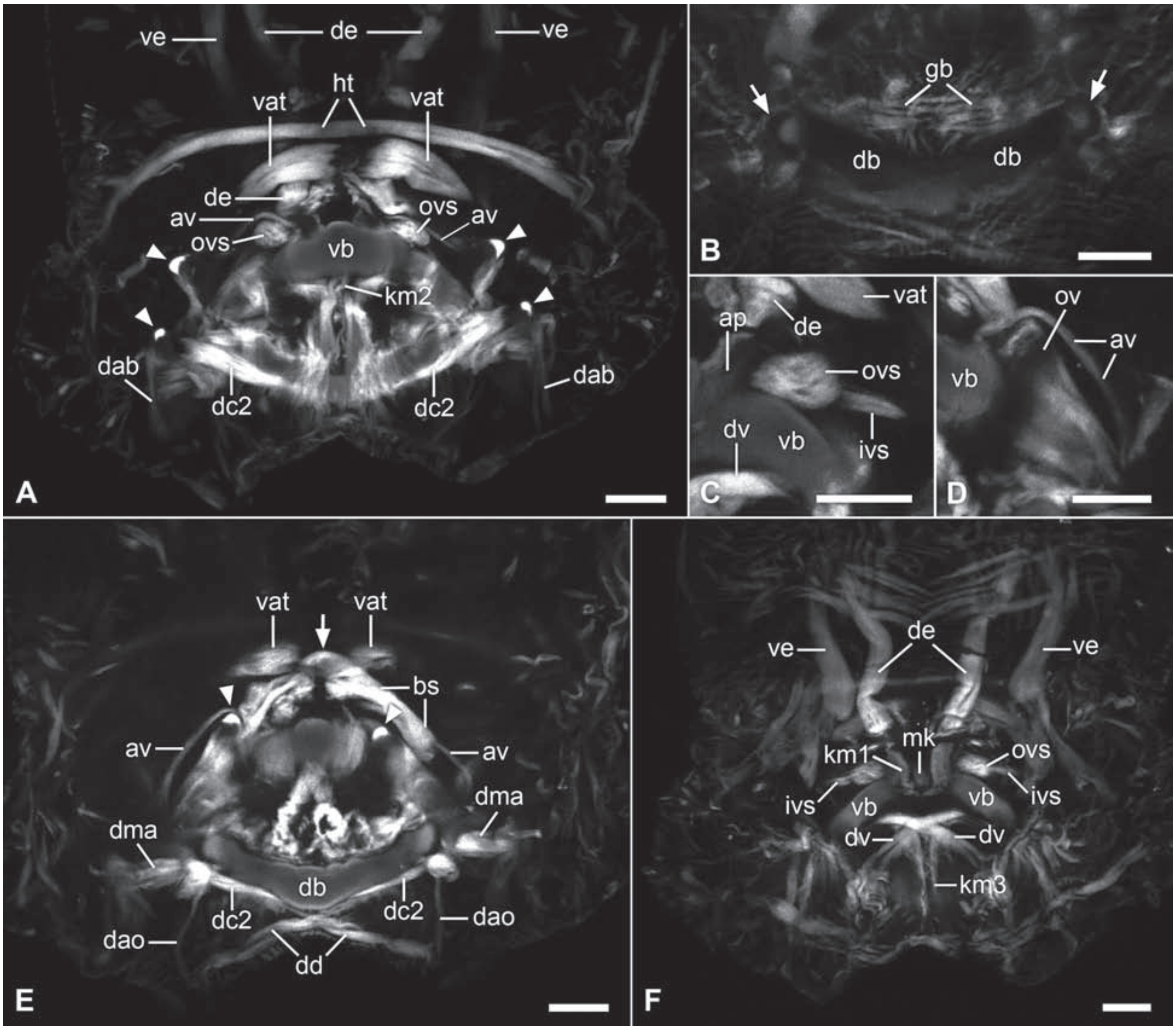


Fig. 3. Haptoral musculature in *L. llewelyni*. Phalloidin-stained whole mounts. Arrowheads point to cap-like structures covering the tips of the anchoral roots. A. Musculature of the haptor. B. Dorsal bar and body-wall musculature. Note that the body-wall musculature above the bar is absent. C. Central portion of the haptor showing muscles attached to the anterior processes (*ivs*) and laminae (*ovs*). D. Central portion of the haptor showing an arch-shaped muscle (*av*) arising from the ventral bar. E. Musculature of the haptor close to the dorsal body side. F. Musculature of the haptor close to the ventral body side. Anterior is towards the top. Scale bars: 10 μm.

Abbreviations: av – arch-shaped muscle; bs – bracket-shaped muscle; dab – muscles coming from dorsal anchor to body wall; db – dorsal bar; dc2 – muscles connecting dorsal bar and anchors; dd – diagonal muscles of dorsal bar; de – dorsal extrinsic muscles; dma – muscles connecting dorsal bar to mid-portion of dorsal anchor; dv – diagonal muscles of ventral bar; gb – grid-like pattern of body-wall muscles; ht – transverse muscle of haptoral constriction; ivs – muscle attached to anterior process; km1–3 – muscle fibres attached to median knob of ventral bar; mk – median knoll; ov – outer root of ventral anchor; ovs – muscle attached to wing-shaped lamina; vat – transverse muscle of ventral anchors; vb – ventral bar; ve – ventral extrinsic muscles.

continue posteriorly close to the ventral side of the haptor along the body midline (Fig. 3A, *km2*). As they course posteriorly, these fibres form a tighter bundle (Figs. 2C,E, 3F, *km3*) and then anchor at the posterior end of the haptor on its ventral side. The ventral bar of *L. llewelyni* and *L. pilengas* has an additional bilateral pair of thin muscles (*av*-muscles, Figs. 2B, 3A,D,E, *av*) attached to a small anterior knob on each side of the bar. Each of these muscles runs laterally arcing over the outer root of the ventral anchor and then

turns posteriorly ending with several small finger-like projections on the inner root of the ventral anchor.

Ligophorus llewelyni and *L. pilengas* have two additional muscles that span widely across the haptor. One of these muscles (*ht*-muscle) is located at the constriction between the haptor and the trunk (Fig. 3A, *ht*). This muscle lies close to the dorsal body wall and runs transversely from one lateral side of the haptoral neck region to the other. The second muscle (*bs*-muscle) is shaped as a triangular bracket (Fig. 3E, *bs*); the tip of this V-shaped bracket points anteriorly and lies at the body midline directly over the tendon-like connection (Fig. 3E, *arrow*) between the *vat*-muscles. The ends of this bracket-shaped muscle terminate near the tips of the inner root of each dorsal anchor, but it seems more likely that they insert on the dorsal body wall in front of the roots rather than directly on the roots by way of tendon-like processes.

The marginal hooks are small and probably do not play any significant role in attachment. The morphology of these hooks and their musculature appears to be identical in all three species. Each of these hooks consists of a straight shaft (Figs. 1D, 2H, *sh*) and a sickle (Fig. 1D, *si*). The sickle has a short base with a small upright thumb (Figs. 1D, 2H, *th*) and a curved blade (Figs. 1D, 2H, *bl*) and is furnished with a ligament (filament loop, Fig. 1D, *fl*). The associated musculature is the same in all 7 pairs of marginal hooks. Two protractor muscles (Fig. 2G, *pm*) extend diagonally from the proximal end of the shaft to the body wall of the haptor, one terminating anterior and the other posterior to the blade. The third muscle (Fig. 2G–H, *sm*) has a distinctly spindle-like shape and runs parallel to the hook between the shaft and the loop. The small size of this muscle makes it impossible to clearly identify its points of insertion on the hook, but it appears to be associated with the loop and its proximal portion is drawn out into a thin thread that terminates at the proximal tip of the shaft.

DISCUSSION

Confocal study of the haptoral musculature in the three species of *Ligophorus* reveals an elaborate arrangement of muscles, with a degree of complexity sufficient enough to effect a set of highly coordinated and precise movements of anchors and connecting bars. These movements are apparently produced by a fairly intricate interplay of forces generated by different haptoral muscles. The situation might be even more complex as the forces exerted by the same muscles may have different effects depending on the orientation of bars and anchors and different muscles can participate in different phases of attachment. Since an accurate interpretation of specific roles of individual muscles in this situation may require a detailed computer-based musculoskeletal simulation, in this paper we provide only a general functional analysis of haptoral musculature.

Ventral and dorsal extrinsic muscles are positioned in such a way that their contraction can rotate the anchors around their points of contact with the connecting bars (arrows 1 in Fig. 4B,D) and these muscles probably play the primary role in initial attachment to the host. However, as argued previously for various groups of monogeneans (see, for instance, Llewellyn 1960 and Halton et al. 1998), the involvement of extrinsic muscles in maintaining the anchors in attached position is probably minimal, because these muscles pass through much of the trunk's length and their sustained contraction would render the body immobile. It should also be noted that if the anchors pivot around a more or less stationary point, their roots must describe a segment of a circle and at a certain point the roots will reverse their direction of movement from anterior to posterior. At this point the contraction of extrinsic muscles will prevent further rotation of anchors making them antagonistic to any muscles involved in pivoting the anchors. For the anchors to continue rotation, they must be pulled by other muscles with extrinsic muscles relaxed. On the other hand, it seems very likely that extrinsic muscles take a major part in producing characteristic exploratory movements of *Ligophorus* involving body flexures and alternating bouts of body contraction and extension; the attached anchors in this case may serve as fixed points against which the muscular force is applied.

Some of the other muscles attached to the anchors may act essentially as catch-muscles holding anchors in a locked position on the host tissues. In case of the ventral anchors, this function could be accomplished by muscles connecting contralateral anchors (*vat*-muscles, arrows 2 in Fig. 4B) and probably by muscle bundles extending to the body wall (*vab*-bundles, arrows 3 in Fig. 4B). Dorsal anchors may be held in position by muscle bundles attached to the body wall (*dab*-bundles, arrows 2 in Fig. 4D). Assistance in securing anchors on gill lamellae can also be provided by the diagonal muscles of the connecting bars

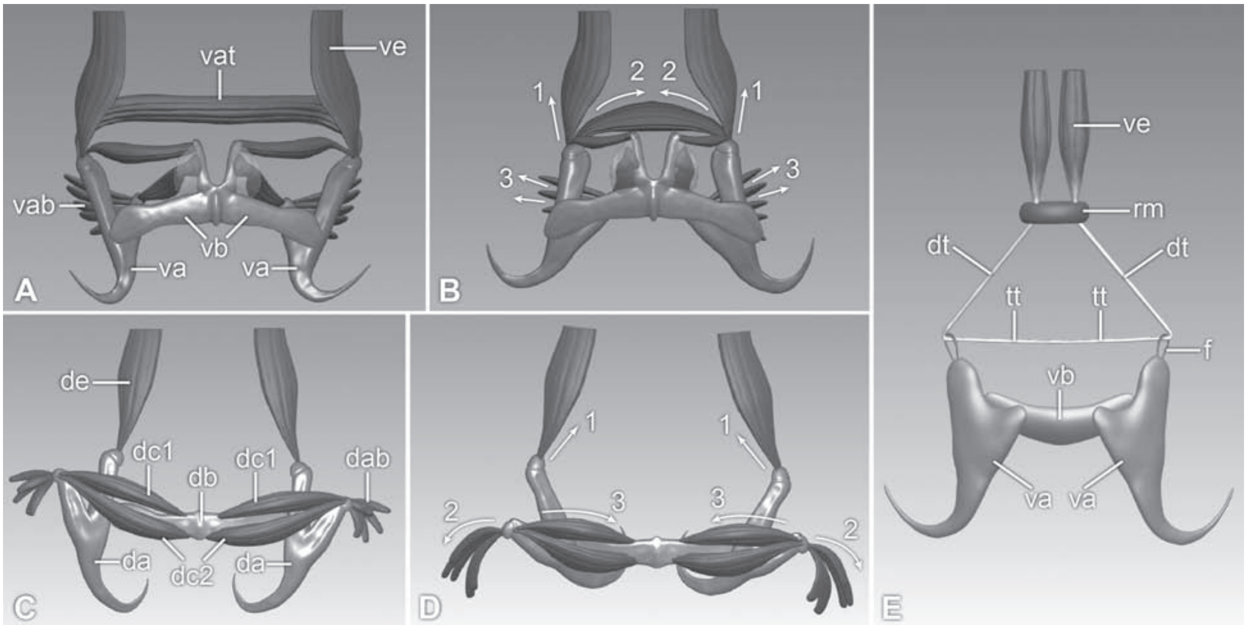


Fig. 4. Diagrammatic representation of the arrangement and possible movements of sclerites and anchoral muscles in *Ligophorus* and *Haliotrema*. Arrows indicate the directions of muscle contraction (see text for further explanations). A. Ventral anchors of *Ligophorus* in unattached position. B. Ventral anchors in *Ligophorus* in attached position. C. Dorsal anchors of *Ligophorus* in unattached position. D. Dorsal anchors in *Ligophorus* in attached position. E. Pulley system of the ventral anchors in *Haliotrema balisticus*. (E) Modified from Kearn 1968.

Abbreviations: da – dorsal anchor; dab – muscles coming from dorsal anchor to body wall; db – dorsal bar; dc1–2 – muscles connecting dorsal bar and anchors; de – dorsal extrinsic muscles; dt – diverging tendinous portions of extrinsic muscles; f – fibrous loop; rm – ring-shaped muscle; tt – transverse tendinous portions of extrinsic muscles; va – ventral anchor; vab – muscles coming from ventral anchor to body wall; vat – transverse muscle of ventral anchors; vb – ventral bar; ve – ventral extrinsic muscles.

(*dd*- and *dv*-muscles) that cross over to the opposite side of the body and end in the immediate proximity of the anchors. The dorsal diagonal muscles (*dd*-muscles) insert on the median side of the anchoral opening and, if they contract with the anchoral sphincter (Fig. 2C, *as*) closed around the dorsal anchors, the blades of the anchors will be brought closer to the dorsal bar and held in a pincer-like grip. The function of the ventral diagonal muscles (*dv*-muscles) might be similar, except that these muscles attach to the body wall laterally to the mid-portion of the anchors and this position probably enables them to pull the tips of the anchors apart in a spreader-like fashion.

Muscles of the ventral anchors connected to the wing-shaped laminae of the ventral bars (*ovs*-muscles) are most likely responsible for the rotation of the ventral anchors in a dorso-lateral direction moving the tips of the opposite anchors away from each other. Muscles attached to the tips of the anterior processes (*ivs*-muscles) probably act as antagonists to the *ovs*-muscles turning the ventral anchors in a ventro-medial direction. It also cannot be excluded that the ventral anchors (especially in *L. kaohsianghsieni*) can be rotated in a medio-ventral direction by the synchronous contraction of *vat*- and *vab*-muscles (arrows 2 and 3 in Fig. 4B); this movement may be accompanied by ventral pivoting of the anchoral blades forcing them deeper into the host tissue. Similarly, the *dc*-muscles of the dorsal anchors can pull the outer roots of the anchors in the medial direction and with the simultaneous contraction of *dab*-bundles can probably drive anchors deeper into the gill lamella (arrows 2 and 3 in Fig. 4D). When the *dc*-muscles are operating independently of *dab*-bundles, they most likely rotate the tips of the ventral anchors laterally away from each other loosening their pincer grip on the host tissue.

The functions of some additional haptoral muscles (*av*, *dao* and *dma* in Fig. 2A,B) are unclear. The *av*- and *dao*-muscles do not seem sufficiently developed to have a marked impact on sclerite movement and, in fact, it is not inconceivable that they are part

of a proprioception system providing sensory input about the orientation of sclerites. The medial end of *dma*-bundle is most likely attached to the dorsal bar, but its lateral attachment cannot be readily identified. Although this bundle might be attached to the outer root of the dorsal anchor, the absence of any corresponding sculpturing on these parts of anchors makes this suggestion implausible. More likely, *dma*-bundles insert laterally on the body wall acting as additional fixator muscles for the dorsal bar.

Wing-shaped lamellae of the ventral bars may serve several functions. Their proximal parts are used as attachment sites for *ovs*-muscles and probably also for *km*-muscles; the latter muscles can probably secure the ventral bar in position when the bar is pulled in the anterior direction. The distal parts of the lamellae may also function as friction dampers to reduce friction for *ovs*- and *km*-muscles. Finally, it is possible that the lamellae act as stop surfaces against which the roots of the rotating ventral anchors can be rested.

The haptoral musculature of *L. llewelyni* and *L. pilengas* differs in some aspects from that of *L. kaohsianghsieni* suggesting that the mechanism of attachment of these two species might also be somewhat different. *Ligophorus llewelyni* and *L. pilengas* have some distinguishing features in the morphology of their muscle bundles: the distal portions of several haptoral muscles give rise to long tendon-like processes and most muscles are thinner compared to *L. kaohsianghsieni*. Another distinguishing feature is the presence of a large V-shaped muscle (*bs*-muscle). The mid-portion of this muscle at the tip of the V appears to be connected to the middle tendinous section of the *vat*-muscles and its distal ends are probably attached to the dorsal body wall. If *vat*- and *bs*-muscles contract simultaneously, they can clamp the posterior portions of the dorsal extrinsic muscles effectively locking the dorsal anchors in the attached position and this clamping of *de*-muscles was, in fact, observed in some of our phalloidin-stained and live preparations.

The marginal hooks in *Ligophorus* probably participate very little in attachment and are expected to have only a vestigial musculature. Two muscles extending diagonally from the proximal tip of the hook to the body wall are undoubtedly used as protractor muscles: when these muscles contract, the blade of the hook is projected outward from the haptor. A similar pair of protractor muscles have been described from *Gyrodactylus rysavyi* Ergens, 1973 (Arafa 2011) and *Macrogyrodactylus congolensis* (Prudhoe, 1957) (Arafa et al. 2007), the only two other species of monogeneans for which the musculature of the marginal hooks is currently known. The musculature of the marginal hooks of *G. rysavyi* and *M. congolensis* has some additional muscles probably involved in articulation and retraction of the hooks. The marginal hooks in *Ligophorus* have only one additional muscle (spindle-shaped muscle) but its function and points of attachment are unclear and its possible homology with the muscles of *G. rysavyi* and *M. congolensis* cannot be adequately assessed.

The identical morphology of haptoral musculature in *L. llewelyni* and *L. pilengas* is consistent with the molecular phylogeny of *Ligophorus* (Blasco-Costa et al. 2012): *L. llewelyni* and *L. pilengas* were shown to have the lowest genetic divergence of all the species included in the analysis. *Ligophorus kaohsianghsieni* differs from these species both in the shape of sclerites and in the haptoral musculature, and it also occupies a more distant position in the phylogenetic tree of 29 Atlantic and Pacific species of *Ligophorus* inferred from 35 morphological characters (Sarabeev and Desdevises 2014). The position of *L. kaohsianghsieni* in this tree, however, is somewhat ambiguous and must be verified by molecular analysis.

The haptoral musculature of *L. llewelyni* and *L. pilengas* with additional muscles, tendon-like muscle extensions and a presumable anchor-locking system consisting of *bs*- and *vat*-muscles has a more complex architecture than that of *L. kaohsianghsieni*. The latter species is markedly larger in size, has a more powerful haptoral musculature and more massive anchors. It also differs from the other two species in its localization on the host: *L. kaohsianghsieni* occupies almost exclusively the second gill arch, while *L. llewelyni* and *L. pilengas* prefer the first arch, although nearly a half of infrapopulations of these latter species are also distributed on the second and third arches (Pronkina et al. 2013). The haptoral musculature of *L. llewelyni* and *L. pilengas* is apparently more specialized and it can be argued that this musculature may have evolved to adapt to the changes in localization on the host and that these changes were accompanied by a progressive decrease in body size.

The haptoral musculature has previously been studied in two other members of the Ancyrocephalidae: in 5 species of *Triacanthinella* Bychovsky et Nagibina, 1968 (Bychovsky and Nagibina 1968) and in *Haliotrema balisticus* (Hargis, 1955) (Kearn 1971).

The morphology of the haptor in *Triacanthinella* and its mode of attachment are strikingly different from those of other ancyrocephalids: most ancyrocephalids, including *Ligophorus*, use their contralateral anchors like pincers or spreaders, but *Triacanthinella* instead clasps the host tissue between ventral and dorsal anchors on each side of the body. The haptoral musculature of *Triacanthinella* appears highly specialized for this mode of attachment making comparison between this and other ancyrocephalids very difficult. By contrast, *Haliotrema* attaches to the gill lamellae in essentially the same way as *Ligophorus* and, in fact, these two genera were shown to be closely related by molecular analysis (Justine and al. 2002). *Haliotrema* and, in fact, most four-anchored monopisthocotyleans whose attachment mechanisms have been studied to date (Llewellyn 1960; Kearn 1966, 1971; Bychovsky and Nagibina 1968; Gerasev 1998) have the anchoral musculature acting on the principle of a pulley system. In *H. balisticus* (Fig. 4E), long tendon-like extensions of both ventral extrinsic muscles run close together into the haptor, pass through a ring-shaped muscle or fibrous ring (Fig. 4E, *rm*), then diverge, thread through fibrous loops (Fig. 4E, *f*) on the inner roots of the ventral anchors and finally join each other at the midline of the haptor between the anchors (Fig. 4E, *tt*). This configuration combines both extrinsic muscles into a single pulley system doubling the forces applied to each anchor.

Although different, the haptoral musculature of *Haliotrema* can be compared to that of *Ligophorus* to establish some tentative homologies. The haptoral musculature of *Ligophorus* is the first one described in Ancyrocephalidae whose mode of action does not involve any pulley-like arrangement and it seems plausible that it represents the plesiomorphic state within this family. If this interpretation is correct, the muscle system of ventral sclerites in *Ligophorus* with both extrinsic and *vat*-muscles attached to the inner roots of the anchors is a more primitive configuration and the same system in *Haliotrema* may constitute a compound structure composed of a pair of extrinsic muscles connected end-to-end to the transverse *vat*-muscles. In this more advanced system, *vat*-muscles may have lost their contractile ability and have been reduced essentially to tendinous extensions of the extrinsic muscles. The loops at the tips of the roots threaded by the tendons may have originated from the actin-rich structures capping the tips of anchoral roots in *Ligophorus*. A possible homology of the ring-shaped muscle in *Haliotrema* is less clear, but it may have arisen from the muscles similar to the *ht*-muscle in *Ligophorus* (Fig. 3A, *ht*) or from one of the circular muscles in the body wall.

Our results show that haptoral musculature can be distinctly different even in the species of the same genus living on the same host and employing the same mode of attachment. Comparison with other ancyrocephalids, such as *Haliotrema*, emphasizes even further a potentially great variability of haptoral musculature in this family. If adequately known in a sufficiently wide range of species, the architecture of haptoral musculature can contribute significantly to the monopisthocotylean taxonomy. Unfortunately, our knowledge of this musculature is still almost entirely lacking and further confocal studies are clearly needed.

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REFERENCES

Arafa S.Z. 2011. Ultrastructure of musculature of the marginal hooklets of *Macrogyrodactylus congolensis*, a monogenean skin parasite from the catfish *Clarias gariepinus*. *Acta Parasitologica,* **56**: 122–130.

Arafa S.Z., El-Naggar M.M., El-Abbassy S.A., Stewart M.T. and Halton D.W. 2007. Neuromusculature of *Gyrodactylus rysavyi*, a monogenean gill and skin parasite of the catfish *Clarias gariepinus*. *Parasitology International,* **56**: 297–307.

Blasco-Costa I., Míguez-Lozano R., Sarabeev V. and Balbuena J.A. 2012. Molecular phylogeny of species of *Ligophorus* (Monogenea: Dactylogyridae) and their affinities within the Dactylogyridae. *Parasitology International,* **61**: 619–627.

Bychovsky B.E. and Nagibina L.F. 1968. *Triacanthinella,* a new genus of marine monogenean parasites of hornfishes. *Parazitologiya,* **2**: 148–158. [In Russian].

El-Naggar M., Arafa S., El-Abbassy S., Stewart M. and Halton D. 2004. Neuromusculature of *Macrogyrodactylus clarii*, a monogenean gill parasite of the Nile catfish *Clarias gariepinus* in Egypt. *Parasitology Research,* **94**: 163–175.

El-Naggar M., Arafa S., El-Abbassy S., Stewart M. and Halton D. 2007. Neuromusculature of *Macrogyrodactylus congolensis*, a monogenean skin parasite of the Nile catfish *Clarias gariepinus*. *Parasitology Research,* **100**: 265–279.

Garcia-Vasquez A., Shinn A. and Bron J. 2012. Development of a light microscopy stain for the sclerites of *Gyrodactylus* von Nordmann, 1832 (Monogenea) and related genera. *Parasitology Research,* **110**: 1639–1648.

Gerasev P.I. 1977. The mechanism of attachment to the host's gills in *Dactylogyrus extensus* and *D. achmerowi* (Monogenoidea). *Parazitologiya,* **11**: 513–519. [In Russian].

Gerasev P.I. 1981. Muscular systems of haptors and the attachment mechanism of some species of *Dactylogyrus* (Monogenea, Dactylogyridae). *Parasitologicheskiy sbornik,* **30**: 190–205. [In Russian].

Gerasev P.I. 1989. Major muscles of hamuli in Amuro-Chinese *Dactylogyrus* (Monogenoidea). Parazitologicheskiye issledovaniya. DVO AN SSSR, Vladivostok: 39–45. [In Russian].

Gerasev P.I. 1998. The family Tetraonchidae (Monogenea): structure and position among monogeneans. *Parazitologiya,* **32**: 544–552. [In Russian].

Halton D. and Maule A. 2004. Flatworm nerve-muscle: structural and functional analysis. *Canadian Journal of Zoology,* **82**: 316–333.

Halton D.W., Maule A.G., Mair G.R. and Shaw C. 1998. Monogenean neuromusculature: some structural and functional correlates. *International Journal for Parasitology,* **28**: 1609–1623.

Justine J.-L., Jovelin R., Neifar L., Mollaret I., Lim L.H.S., Hendrix S.S. and Euzet L. 2002. Phylogenetic positions of the Bothitrematidae and Neocalceostomatidae (Monopisthocotylean monogeneans) inferred from 28S rDNA sequences. *Comparative Parasitology,* **69**: 20–25.

Kearn G. 1966. The adhesive mechanism of the monogenean parasite *Tetraonchus monenteron* from the gills of the pike (*Esox lucius*). *Parasitology,* **56**: 505–510.

Kearn G. 1971. The attachment of the ancyrocephalid monogenean *Haliotrema balisticus* to the gills of the trigger fish, *Balistes capriscus* (= *carolinensis*). *Parasitology,* **63**: 157–162.

Llewellyn J. 1960. Amphibdellid (monogenean) parasites of electric rays (Torpedinidae). *Journal of the Marine Biological Association of the United Kingdom,* **39**: 561–589.

Plaisance L., Littlewood D.T.J., Olson P.D. and Morand S. 2005. Molecular phylogeny of gill monogeneans (Platyhelmintes, Monogenea, Dactylogyridae) and colonization of Indo-West West Pacific butterflyfish hosts (Perciformes, Chaetodontidae). *Zoologica Scripta,* **34**: 425–436.

Pronkina N.V., Dmitrieva E.V., Gerasev P.I. and Gibson D.I. 2013. The distribution of species of *Ligophorus* Euzet et Suriano, 1977 (Ancyrocephalidae) on the gills of their hosts: coexistence and species divergence. Abstracts of 7th International Symposium on Monogenea, August 4–9, 2013. Rio de Janeiro: 81.

Sarabeev V. and Desdevises Y. 2014. Phylogeny of the Atlantic and Pacific species of *Ligophorus* (Monogenea: Dactylogyridae): Morphology vs. molecules. *Parasitology International,* **63**: 9–20.

Šimková A., Matejusová I. and Cunningham C.O. 2006. A molecular phylogeny of the Dactylogyridae *sensu* Kritsky & Boeger (1989)(Monogenea) based on the D1–D3 domains of large subunit rDNA. *Parasitology,* **133**: 43–53.

Šimková A., Plaisance L., Matějusová I., Morand S. and Verneau O. 2003. Phylogenetic relationships of the Dactylogyridae Bychowsky, 1933 (Monogenea: Dactylogyridea): the need for the systematic revision of the Ancyrocephalinae Bychowsky, 1937. *Systematic Parasitology,* **54**: 1–11.

Valigurová A., Hodová I., Sonnek R., Koubková B. and Gelnar M. 2011. *Eudiplozoon nipponicum* in focus: monogenean exhibiting a highly specialized adaptation for ectoparasitic lifestyle. *Parasitology Research,* **108**: 383–394.

Zurawski T., Mousley A., Mair G., Brennan G., Maule A., Gelnar M. and Halton D. 2001. Immunomicroscopical observations on the nervous system of adult *Eudiplozoon nipponicum* (Monogenea: Diplozoidae). *International Journal for Parasitology,* **31**: 783–792.

Zurawski T., Mousley A., Maule A., Gelnar M. and Halton D. 2003. Cytochemical studies of the neuromuscular systems of the diporpa and juvenile stages of *Eudiplozoon nipponicum* (Monogenea: Diplozoidae). *Parasitology,* **126**: 349–357.

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